specifically integrates at an FRT within the genome of said mammalian cell and **[,when combined]** combines in reading frame with said first DNA, wherein the combination of said first and second DNAs provides a functional gene.

45. (Twice Amended) A method according to Claim 42, wherein said FLP recombinase is provided by **[a]** an FLP expression vector.

46. (Twice Amended) A method according to Claim 45, wherein the expression of FLP recombinase **[by said FLP expression vector]** is subject to regulatory control.

48. (Twice Amended) A method according to Claim 42 **[wherein the FLP recombination target site in the genomic DNA of said mammalian cell is so positioned in a gene of interest that the introduction of additional DNA sequences therein will inactivate]** , further comprising contacting said transfected mammalian cell with a second DNA comprising a nucleotide sequence containing at least one FRT, in the presence of an FLP recombinase, wherein said second DNA specifically integrates at an FRT within the genome of said mammalian cell and combines with said first DNA, wherein the combination of said first and second DNAs, prevents expression of the gene of interest.

Cancel claims 27, 47, and 49-55 without prejudice.

REMARKS

The novel site-specific recombination system of the present invention provides an artisan with the ability to target the integration of transfected DNA to a **specific** chromosomal site in a mammalian host cell at **frequencies exceeding** those of both

random and other site-specific integration systems. Additionally, this novel recombination system allows for **immediate confirmation** and analysis of the recombination event. Applicants' recombination system is distinctive in its **precision** and **predictability**, providing methods which enable an artisan to **routinely create** a **functional** translational reading frame at any desired site of integration.

Claims 25-28 and 42-55 stand variously rejected under 35 U.S.C. § 112, first and second paragraphs, under § 102(b) as anticipated by Golic et al., and under § 103 as unpatentable over Sauer (U.S. '317) taken with Golic et al., and further in view of Palmiter et al. Claims 25, 26, 28, 42-46 and 48 have been amended to define Applicants' invention with greater particularity. No new matter is introduced by the amendments. Support for the amendments is found throughout the specification of the application as originally filed. Claims 27, 47, and 49-55 have been canceled without prejudice to decrease the number of issues currently under consideration.

The aforementioned amendments were made to present the claims in condition for allowance, or alternatively, to present the claims in better condition for appeal. Such amendments are necessary to clarify the scope of the invention in response to issues raised in the June 3, Final Office Action and were not made earlier because Applicants' were of the opinion that the clarity and meaning intended was apparent in view of Applicants' amendment (filed 03/09/93) in response to the preceding Office Action (Paper No. 15, mailed 09/26/92).

Accordingly, claims 25, 26, 28, 42-46 and 48 remain under examination.

I. Rejections Under 35 U.S.C. §112, First Paragraph

The specification stands objected to, and claims 25-28 and 42-55 stand similarly rejected under 35 U.S.C. §112, first paragraph. The Examiner asserts that the specification allegedly fails to provide a reasonable written description, enablement and best mode for practicing the claimed invention. This rejection is respectfully traversed.

Applicants respectfully submit that the specification and claims fully satisfy the written description, enablement and best mode requirements of 35 U.S.C. § 112, first paragraph. Applicants' claims and specification clearly and reasonably convey to a person of ordinary skill in the art that Applicants had, indeed, successfully practiced and possessed, at that time, methods enabling precise targeting and site-specific integration or excision of DNA within the genome of a mammalian host cell.

The specification clearly provides sufficient direction and guidance on how to practice the invention, as well as working examples such that one skilled in the art could reproduce and practice Applicants' invention. The specification defines the mammalian host cells used in Applicants' invention as including all members of the order Mammalia, for example, human, mouse, rat, monkey, hamster and the like (page 15, lines 19-23). Applicants demonstrate the versatility of the methods claimed utilizing human cell line 293, monkey cell line CV-1 and murine cell line F9 in Examples I-III (pages 17-27). The mammalian cell lines used in these examples are well known, characterized and commonly available to those of ordinary skill in the art.

Contrary to the Examiner's assertion that the specification fails to provide an example descriptive of the

process defined by claims 25-28 (Paper No. 18, page 2, lines 9-10), Examples I-III clearly detail the process defined by claims 25-28. Specifically, claims 25-28, as amended, provide methods that enable a person of skill in the art to precisely target a locus in the genome of a mammalian cell to which DNA(s) is to be integrated. Examples I-III detail FLP-mediated recombination of extrachromosomal DNA, FLP-mediated removal of intervening sequences, as well as FLP-mediated recombination of FRTs on an extrachromosomal molecule with an integrated FRT in a variety of mammalian cell lines.

In addition to the processes described in Examples I-III, Applicants provide protocols for construction of FLP expression vectors (page 19, lines 20-36). Moreover, the nucleic acid and amino acid sequences of the FLP recombinase contemplated for use in the claimed methods are also provided (pages 28-34), as well as references and preferred strains of Saccharomyces cerevisiae from which FLP recombinase can be obtained (page 11, lines 1-20). Examples of the various DNAs/genes of interest are those that provide a readily analyzable functional feature to the host cell, such as, visible markers, selectable markers and other genes that function to alter the phenotype of the host cell (see page 12, lines 18-27). Working examples utilizing visible marker gene ß-galactosidase and selectable marker gene conferring neomycin resistance are provided in Examples I-III.

Furthermore, Applicants detail numerous manipulations and combinations of DNA(s) used to construct a functional expression unit (page 12, lines 28-36; page 13, lines 1-36), as well as means for disassembling a functional expression unit (page 14, lines 7-36; page 15, lines 1-3) to achieve the desired result. Desired results include production of a complete functional gene, production of a functional hybrid gene,

disruption of expression of a gene of interest, gene inactivation, disassociation of the gene of interest from the transcriptional control of the promoter with which it is normally associated and the like (page 13, lines 3-36; page 14, lines 1-36; page 15, lines 1-3).

Applicants describe the requirements for constructing an FRT and various modifications which provide certain advantages, dependent upon the specific circumstances in which they are employed (page 11, lines 21-37; page 12, lines 1-4). A specific example of construction of an FRT and its insertion into a gene of interest is described in Example I (page 17, lines 15-36; page 18, lines 1-30; Figures 1-2), in Example II (page 22, lines 14-25) and in Example III (page 25, lines 25-29; page 26, lines 13-30; Figure 2).

Constructing expression vectors, assembling functional expression units, designing and constructing FRTs, and introducing such informational structures into the genome of a mammalian host cell are among the basic tools utilized daily in the art of molecular and cellular biology. Applicants provide examples of the application of such standard techniques to the practice of the present invention, i.e., transfection, microinjection, electroporation, retroviral vector transduction (page 15, lines 4-14), and conditions suitable for site specific recombination of DNA (page 15, lines 30-36; page 16, lines 1-6), all of which are conventional in the art and would be commonplace to a person of skill in the art.

Clearly, Applicants have detailed the methods of the invention in the specification such that a person of ordinary skill in the art can reproduce and practice Applicants' invention. Therefore, Applicants respectfully submit that the

claims under examination are fully enabled. Further, Applicants provide working examples of the present invention in specific human, monkey and mouse cell lines. Therefore, it is respectfully submitted that Applicants' specification unquestionably sets forth the best mode of practicing the invention contemplated by Applicants at the time of filing.

The Examiner has set forth specific inquiries under the 35 U.S.C. § 112, first paragraph rejection (see Paper No. 18, page 2, lines 6-27; page 3, lines 1-22), the responses to which are encompassed generally by Applicants' amendment of the claims and remarks set forth above. However, in order to expedite prosecution of the subject invention, each inquiry is individually addressed below.

The Examiner's concerns regarding the term "non-human host organism" have been rendered moot by Applicants' amendment of the claims to recite "mammalian host cells".

With respect to the inquiry as to the placement of the FRT (FLP Recombination Target site), the FRT can be inserted within a "gene of interest" at a location such that the nucleotides encoding the FRT, at the artisan's option, either do or do not disrupt the expression of the gene of interest. Thus, the FRT insertion site **is** "predetermined" with respect to the intended result of the recombination event.

The pNEOßGAL reporter plasmid (derived from pFRTßGAL), was constructed by Applicants to allow characterization of FLP-mediated modifications of DNA in mammalian cell lines. pFRTßGAL, containing the bacterial ß-galactosidase coding sequence (gene of interest), was modified by inserting the FRT immediately 3' to the ß-gal translational start codon, while preserving the ß-gal

translational reading frame. pNEOßGAL results from a restriction digest of pFRTßGAL with an enzyme that recognizes the "spacer" region in the FRT and the insertion of a complementary fragment, which creates intact FRTs on each side of the insert. This insertion disrupts the ß-gal translational reading frame and renders the ß-gal transcriptional unit inactive. Subsequent exposure of pNEOßGAL to FLP recombinase excises the disruptive insertion, re-creating the parental pFRTßGAL plasmid and restoring ß-gal expression (see page 17, line 15 through page 20, line 28 and Figure 2).

Thus, the specific insertion site for the FRT sequence, with respect to the sequence of the "gene of interest" is a function of the result intended by the insertional modification, i.e., production of a complete functional gene, production of a functional hybrid gene, disruption of expression of a gene of interest, gene inactivation, or disassociation of the gene of interest from the transcriptional control of the promoter with which it is normally associated.

With respect to the inquiries as to how specific targeting is accomplished, the construct (containing the "gene of interest" and the FRT) is provided in a vehicle that facilitates entry into the host cell. Once the initial FRT integrates within the transfected host cell's genome, all subsequent introductions of DNAs having FRT(s) will be targeted specifically to this site in the presence of FLP recombinase. In the absence of FLP-mediated recombination, activation of the expression unit will not produce a functional protein product. Thus, Applicants' expression system, unlike other gene expression systems which are typically governed by a single set of control sequences, is under the binary control of cis- or trans- acting sequences.

In view of the above remarks, Applicants respectfully request reconsideration and withdrawal the rejection of the claims under 35 U.S.C. §112, first paragraph.

II. Rejections Under 35 U.S.C. §112, Second Paragraph

Claims 25-28 and 42-55 stand rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants respectfully traverse the Examiner's rejection.

These concerns have been rendered moot as Applicants have amended claims 25, 26, 28, 42-46 and 48 and have canceled claims 27, 47 and 49-55 in order to further prosecution of the subject invention. Specifically, in claim 25, Applicants have replaced the reference to "non-human host organism" with "a mammalian host cell" and have deleted step (iii). This amendment clarifies that the genome of the mammalian host cell is genetically altered. The reference to "portion" in claim 43 has been clarified. Specifically, claim 43, as amended, refers to the placement of FRTs within a **functional portion**, i.e., regulatory sequences associated with gene expression and/or coding region sequences, of a gene of interest. The motivation behind the precise targeting, site-specific methods of gene modification is to affect, in some way, the functional expression of the gene product. Therefore, the FRT "target sites" will be inserted within the functional portion of the gene of interest. The exact locus will depend upon whether expression or inactivation is desired.

Claims 44 and 48, as amended, clearly define the procedure for inserting a second DNA into a mammalian host cell

containing a previously inserted first DNA. Applicants detail first and second DNAs and combinations thereof on page 12, lines 28-36; page 13, lines 1-36; page 14, lines 1-36; and page 15, lines 1-3.

III. Rejections Under 35 U.S.C. § 102(b)

Claims 25-28 stand rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Golic et al.

Applicants' claims, as amended, require a "mammalian host cell". In contrast to the mammalian host cells of Applicants' invention, the host organism described in Golic et al., is an insect species, Drosophila melanogaster. The Golic et al. reference, therefore, does not anticipate the present invention as described in the claims. Entry of the amendments to claims 25, 26 and 28 are respectfully submitted to be proper in response to the Examiner's request for clarification of the metes and bounds of Applicants' invention.

In view of the above remarks, Applicants respectfully request the Examiner to reconsider and withdraw the rejection of the claims under 35 U.S.C. § 102(b).

IV. Rejections Under 35 U.S.C. § 103

Claims 25-28 and 42-55 stand rejected under 35 U.S.C. § 103 as allegedly being unpatentable over Sauer (U.S. '317) taken with Golic et al. Applicants respectfully disagree with the Examiner's assertion that "although Sauer does not explicitly disclose the use of DNA coding for FLP and FRT, it would have been obvious to one of ordinary skill in the art to use DNA coding for FLP and FRT in vectors for transforming D.melanogaster

because Golic et al. disclosed site specific recombination in D. melanogaster with DNA coding for FLP and FRT . . ." (Paper No. 18, page 4, lines 20-24). Further, Applicants disagree that the concluding sentence in Golic et al., specifically "we expect that it will work in other organisms as well. . ." provides "motivation to one of ordinary skill in the art to combine the teachings of Sauer" with the teachings of Golic et al.

Applicants' invention, as defined by claims 25 and 42, distinguishes over the art by providing methods for integrating and recombining DNA at a specific, predetermined site in the genome of a mammalian host cell. Applicants' conditional recombination system utilizes a **eukaryotic** site-specific recombinase, FLP, from Saccharomyces cerevisiae and its recombination targets, FRTs. Applicants' invention, in one aspect, involves a **gain of function** system wherein non-functional gene sequences are precisely rearranged enabling the expression of a functional gene product.

An advantage of Applicants' recombination system over prior art systems is that it provides one with the ability to target the integration of transfected DNA to a **specific** chromosomal site in a mammalian host cell at **frequencies exceeding** those of both random and other site-specific integration systems. Another advantage of Applicants' recombination system is that it allows for **immediate confirmation** and analysis of the recombination event. A further advantage of Applicants' recombination system is its **precision** and **predictability**, allowing a person of skill in the art to **routinely create** a **functional** translational reading frame at the site of integration.

In contrast, the recombination system taught by Sauer utilizes a **prokaryotic** protein from bacteriophage P1, Cre, and its recombination targets, lox, to effect recombination and integration of DNA into the chromosomes of the non-mammalian yeast species S. cerevisiae and into target sites on extrachromosomal plasmids in mammalian cells. Unlike Applicants' **eukaryotic** recombination system which is precise and predictable, Sauer's prokaryotic protein fails to efficiently catalyze recombination between separate target sites integrated in the chromosome of a mammalian host cell. In mammalian cells, Sauer's prokaryotic recombination system is **not** precise and does **not** allow for the routine creation of a functional translational reading frame at the site of integration. In contrast to Applicants' eukaryotic system, neither the nuclear membrane nor the packaging of the prokaryotic lox target sequences into chromatin prevent spontaneous recombination of the target sites.

Golic et al. report a **loss of function** system. The Golic system utilizes a **positive genetic marker**, i.e., the white gene (typically present in Drosophila genome). When the wild-type gene is present in the Drosophila genome, its expression results in red eyes, if the white gene is absent or mutated, its failure to be expressed results in eyes without pigment. Using P element-mediated germline transformation[1], Golic et al. introduced a **functional** white gene into D. melanogaster having a

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[1]. P elements are transposable DNA elements that are highly mobile in the germline of D. melanogaster. The subjugation of these sequences as specialized vectors facilitates the integration of a DNA sequence at diverse sites in the chromosomes of D. melanogaster.

negative genetic background[2] (the functional gene is absent from the genome or is mutated and is not expressed). Upon exposure to FLP recombinase, the integrated **functional** gene is inactivated and its expression terminated, resulting in a loss of function, generating a **negative signal**, i.e., no expression.

In contrast, Applicants' invention is a **gain of function** system. This system utilizes a **negative genetic marker**, i.e., the ß-galactosidase gene (typically absent in mammalian cells). Applicants introduce a negative form, i.e., **non-functional**[3] construct of the ß-galactosidase gene into mammalian cells. Upon exposure to FLP recombinase, the integrated non-functional gene sequences are precisely[4] rearranged in the correct reading frame such that the gene becomes **functional**. Applicants' FLP-mediated recombination system results in a gain of function, generating a **positive signal**, i.e. expression of active ß-gal enzyme. A positive signal is more desirable than a negative signal because it confirms that the non-functional integrated gene is precisely recombined in a correct reading frame such that it becomes functional and expresses active gene product. In contrast, a negative signal in a loss of function system merely acknowledges that a gene is unable to express its

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[2]. The Golic et al. loss of function system requires an organism with a positive genetic marker and a negative genetic background. These conditions exclude virtually all mammalian cells.

[3]. The ß-gal gene is rendered non-functional via the insertion of FRT sequences which disrupt the ß-gal coding sequence.

[4]. The Golic et al. and Sauer systems do not require this stringent condition. No one, at the time of the subject invention, had a system that could recombine with such precision.

product. A negative signal does not provide any information regarding the alleged recombination event.

There was no teaching or suggestion in the art that site-specific recombinases could accurately and routinely create a functional translational reading frame, as shown by Applicants' FLP-mediated gain of function recombination system. Accordingly, it was clearly unknown at the time of Applicants' invention, that a **eukaryotic** site-specific recombinase, FLP (from Saccharomyces cerevisiae) and its recombination targets, FRTs, could be successfully employed in a **gain of function** system wherein non-functional gene sequences are precisely rearranged to provide the expression of a functional gene product.

Golic et al. do not teach or suggest a method for **precisely** targeting a specific site in the genome of a mammalian cell in order to rearrange and recombine specific target sequences, wherein the targeted sequences, as recombined, express a functional gene product. Golic et al., in fact, teach away from recombining specific target sequences in order to express a functional gene (gain of function system). Indeed, Golic et al. teach a **loss** of function system, wherein a functional gene is **inactivated**. Further, the Golic et al. system is limited to organisms, such as Drosophila, having a positive genetic marker and a negative genetic background. Additionally, the functional gene is introduced into the germline of the organism using a transformation system exclusive to Drosophila. These parameters are not amenable to mammalian host systems. It is obvious, therefore, that having only tested the FLP recombinase system in Drosophila, the statement "we expect that it will work in other organisms as well" only applies to other host systems meeting the above-identified parameters, thus, precluding any reasonable expectation of success in **mammalian** host systems. Therefore, it

is clear that Golic et al. fail to provide any motivation to a person of skill in the art to modify Golic et al.'s loss of function system for use in mammalian host cells as required by the present claims.

A further indication of the lack of motivation to combine Sauer and Golic et al. is the fact that Golic et al. **chose** D. melanogaster rather than a mammalian host to test the FLP recombination system. Golic and associates were, indeed, familiar with the Sauer prokaryotic recombinase system and knew that it did **not** efficiently catalyze recombination between target sites in mammalian cells (Golic et al., Cell 59:499, column 2 (1989)). In choosing the Drosophila host system, it is apparent that the significance and advantages of the subject invention were not recognized by Golic et al.

A discussion of the Palmiter et al. secondary reference is moot in view of the fact that the combination of the Sauer and Golic et al. primary references neither teach nor suggest the novel recombination system of Applicants' invention. The Palmiter reference does not cure the inadequacies of the primary references.

The subject invention is not rendered obvious by the art cited by the Examiner. The teachings relied upon by the Examiner would not motivate one of ordinary skill in the art to combine the references. The Examiner has employed improper hindsight analysis, using the teachings of the subject invention as the motivation to combine the cited references. The mere fact that a prior art system could be modified to produce the claimed invention would not have made the modification obvious unless the prior art suggested the desirability of the modification. In re Gordon, 221 USPQ 1125, 1127 (Fed. Cir. 1984).

The Examiner has failed to offer any evidence for the conclusion that the manipulations contemplated by the present invention would have been obvious. Applicants contend that the Examiner has improperly used hindsight analysis to provide the motivation to combine the cited references. Thus Applicants assert that the Examiner has failed to establish a prima facie case of obviousness.

In view of the remarks set forth above, Applicants respectfully request reconsideration and withdrawal of the rejection of the claims under 35 U.S.C. §103.

III. Summary

In summary, the claims as amended, in light of the remarks herein, are submitted to be in condition for allowance. If any questions or issues remain, the Examiner is invited to contact the undersigned the telephone number set forth below so that a prompt disposition of this application can be achieved.

Respectfully submitted,

10/13/93
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